REMARKS

Claims 120-138 are pending. Claims 123-131 and 135-138 are withdrawn from consideration. The Office objects to claim 134. Claims 120-122 and 132-134 are rejected under 35 U.S.C. § 112, first paragraph, for lack of written description and lack of enablement. Claims 132-134 are rejected under 35 U.S.C. § 102(b) for lack of novelty over Umezawa et al. (U.S. Patent Appln. Publn. No. 2002/0142457; hereinafter "Umezawa"). Finally, the Office objects to the abstract and to the specification. By this reply, Applicants cancel claims 123-125, 127, 129, 131, and 136-138, amend claims 120, 121, and 132, add new claims 139-145, amend the specification and abstract, and respond to each of the Office's objections and rejections.

Telephonic Interview

Applicants wish to thank Examiner Dunston for the courtesy of a telephonic interview, which was conducted on February 1, 2010, with Dr. Todd Armstrong. During the interview, the written description and enablement rejections of claims 120-122 and 132-134 and the anticipation rejection of claims 132-134 were discussed. Applicants have amended independent claims 120 and 132 as was suggested by Examiner Dunston. The Office acknowledged that the present amendments would likely address all of the present objections and rejections.

Support for the Amendment

The Specification is amended to incorporate sequence identifiers and to address other formalities. The Abstract is amended to reduce the word count.

Support for the amendment to claims 120, 121, and 132, and for new claims 139-145, is found in the specification at, e.g., page 14, lines 21-27, page 24, lines 12-19, page 28, lines 25-27, and Figure 13. No new matter is added by the amendments.

Objection to the Specification

The Office objects to the number of words in the Abstract. The Abstract has been truncated to fewer than 150 words. This objection can be withdrawn.

The Office also objects to the Specification for reciting sequences without a sequence identifier. The Specification has been amended to incorporate sequence identifiers. This objection can be withdrawn.

The Office objects to the Specification for including a typographical error on page 4, a hyperlink on page 16, and an unidentified trademark on page 54. Each of these objections has been addressed by the present amendment.

Objection to Claim 134

The Office objects to claim 134 because the term "picornavirus" is misspelled. Claim 134 has been amended to remove this term. This objection can be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph

Written Description

The Office rejects claims 120-122 and 132-134 under 35 U.S.C. § 112, first paragraph, for lack of written description, stating.

> Given the very large genus of RTEF-1 polypeptides and nucleic acid molecules encompassed by the rejected claims, and given the limited description provided by the prior art and specification with regard to structure-function correlation or a representative number of species, the skilled artisan would not have been able to envision a sufficient number of specific embodiments that meet the functional limitations of the claims to describe the broadly claimed genus. (Office Action, p. 11.)

Applicants respectfully disagree, but in an effort to expedite prosecution of the present claims, Applicants have amended independent claims 120 and 132 to recite an RTEF-1 polypeptide having at least 85% sequence identity to the sequence of SEQ ID NO. 7; the genus encompasses species having ~65 mutations or deletions in the RTEF-1 amino acid sequence. For the reasons discussed below, the present specification satisfies the written description requirement with respect to this genus of RTEF-1 polypeptides, and nucleic acids encoding the same.

As was discussed during the telephonic interview, Applicants' specification teaches that the DNA binding domain at the amino-terminal end of RTEF-1 (amino acids 24-98) has structural significance for biological function (see, e.g., p. 15, lines 4-6). As is discussed in Ueyama et al. (J. Biol. Chem. 275:17476-17480, 2000), which is cited in the present specification, the DNA binding domain is highly conserved among the transcription enhancer factor-1 (TEF-1) multigene family of transcription factors; human RTEF-1 shares 100% sequence identity with human TEF-1 within the DNA binding domain (see Fig. 1 of Ueyama et al.). Within the RTEF-1 subfamily, this region is also highly conserved. As evidence, Applicants note that the chick RTEF-1 shares 98% sequence identity with the human sequence within this region.

In addition, Applicants direct the Office to Appukuttan et al. (Investigative Ophthalmology & Visual Science 48:3775-3782, 2007; a copy of which is provided), which shows that a human RTEF-1 splice isoform lacking 65% of the full-length content (a loss of 285

residues) retains the ability to promote expression of VEGF. This splice isoform, which includes the DNA binding domain, has only 149 amino acids of the normal 434 amino acids and exhibits "greater activation of expression from the VEGF promoter than other human isoforms" (Appukuttan et al., p. 3781, 2nd col.). Thus, a significant portion of the carboxy-terminal residues of RTEF-1 (the 285 amino acids encoded by exons 5, 8-11, and portions of 7 and 12) can be removed without loss of the ability to promote VEGF expression and angiogenesis.

During the telephonic interview, the Office acknowledged that, given the evidence presented above, claims 120-122, 132-134, and 139-145, as presently amended satisfy the written description requirement. Thus, Applicants respectfully request that the rejection of claims 120-122 and 132-134 be withdrawn.

Enablement

The Office also rejects claims 120-122 and 132-134 for lack of enablement. As was discussed during the telephonic interview, Applicants have amended independent claims 120 and 132 to recite that the method involves providing "within or adjacent to a tissue in need thereof" in a mammal a therapeutically effective amount of an RTEF-1 polypeptide having at least 85% sequence identity to the sequence of SEQ ID NO: 7, or a nucleic acid molecule encoding the polypeptide. Applicants explained that the methods could be applied to the tissue of a patient before a hypoxic or ischemic condition, and thus, should not be limited to treatment of hypoxic or ischemic tissue. For example, the patient may be treated prior to organ transplantation to increase the vasculature surrounding the organ so as to improve the outcome following organ transplantation. Applicants respectfully submit that present independent claims 120 and 132, and claims dependent therefrom, are fully enabled with respect to the mode of administration.

In addition, the Office states that the specification does not enable the use of any polyomavirus, papillomavirus, or picornavirus vectors. Applicants have removed reference to the use of a picornavirus vector, but respectfully disagree that the use of a polyomavirus or papillomavirus vector is not enabled by the present specification. Several publications describe the generation or use of polyomavirus and papillomavirus vectors (see, e.g., Touzé et al., J. Gen. Virol. 82:3005-3009, 2001; Sasnauskas et al., Intervirology 45:308-317, 2002; Tegerstedt et al., Anticancer Res. 25:2601-26018, 2005; Georgens et al., Curr. Pharm. Biotechnol. 6:49-55, 2005; Krauzewicz et al., Gene Therapy 7:2122-2131, 2000; Krauzewicz and Griffin, Cancer Gene Therapy: Past Achievements and Future Challenges, Vol. 465, pp. 73-82, Ed. Habib Kluwer Academic/Plenum Publishers, New York, 2000; Khan and Sverdrup, Methods in Molecular Medicine, In Gene Therapy Protocols, Humana Press, Chap. 7:117-125, 1997; Tammur et al., BMC Mol. Biol. 3:5, 2002; Eisenberger et al., Gene Ther. Mol. Biol. 9:371-376, 2005; and Sarver et al., Mol. Cell. Biol. 1:486-496, 1981; copies of the abstracts are provided.) Thus, the use of these vectors in the present method is well within the purview of one of skill in the art. Applicants respectfully submit that present independent claims 120 and 132, and claims dependent therefrom, are fully enabled with respect to the use of polyomavirus and papillomavirus vectors.

Finally, Applicants have amended independent claim 132 to remove reference to "preventing" hypoxia.

For all of the reasons discussed above, Applicants respectfully request that the rejection of claims 120-122 and 132-134 for lack of enablement be withdrawn.

Rejection under 35 U.S.C. § 102(b)

Claims 132-134 are rejected under 35 U.S.C. § 102(b) for lack of novelty over Umezawa. As acknowledged by the Office during the telephonic interview, the present amendment to independent claim 132 overcomes this rejection. In particular, Umezawa fails to teach or suggest providing an RTEF-1 polypeptide having at least 85% sequence identity to the sequence of SEQ ID NO:4 within or adjacent to a tissue in need thereof in a mammal to treat or reduce hypoxia.

Instead, Umezawa describes only the administration of "cells having the potential to differentiate into cardiomyocytes...as therapeutic agents for cardiac regeneration or for heart diseases" (see ¶ [0151]). Thus, Umezawa fails to teach or suggest a method for treating or reducing hypoxia in a tissue in need thereof in a mammal. Accordingly, Applicants respectfully request that the rejection of claims 132-134 for lack of novelty over Umezawa be withdrawn.

CONCLUSION

Applicants respectfully submit that present claims 120-122, 132-134, and 139-145 are in condition for allowance, and such action is respectfully requested.

A petition to extend the period for replying for one (1) month, to and including February 28, 2010, is submitted herewith. Applicants authorize the Office to deduct the fee required by 37 C.F.R. § 1.17(a) for the petition from Deposit Account No. 03-2095.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Todd Armstrong, Ph.D.
Reg. No. 54,590

Date: 16 February 2010

for Kristina Bieker-Brady, Ph.D.
Reg. No. 39,109

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Identification of Novel Alternatively Spliced Isoforms of RTEF-1 within Human Ocular Vascular Endothelial Cells and Murine Retina

Binoy Appukuttan, Trevor J. McFarland, Michael H. Davies, La-ongsri Atchaneeyasakul, Yi Zhang, Bobby Babra, Yuzhen Pan, James T. Rosenbaum, Ted Acott, Michael R. Powers, and J. Timothy Stout

Purpose. Identification of transcription factors that regulate the transcription of the vascular endothelial growth factor (*VEGF*) gene may facilitate understanding of the etiology and progression of ocular neovascular diseases. The purpose of this study was to determine whether transcriptional enhancer factor 1-related (RTEF-1) was present within ocular vascular endothelial cells and whether it played a role in the control of the transcription of the *VEGF* gene.

Methods. Primary cultures of human retinal vascular endothelial cells (RVECs) were maintained under normoxic or hypoxic conditions before isolation of mRNA. RT-PCR was performed to detect *RTEF-1* transcripts. Amplified products were cloned into an expression plasmid. Human VEGF promoter and deletion constructs were cloned into a pSEAP reporter vector. Various RTEF-1 isoforms and VEGF promoter constructs were coelectroporated into human cells, and reporter expression levels were determined. Retinal tissue from a mouse model of retinopathy of prematurity (ROP) was analyzed by RT-PCR for the presence of *RTEF-1* transcripts.

Results. Full-length 1305-bp and novel 936-bp *RTEF-1* transcripts were identified in cultured human RVECs under normoxic conditions. A novel 447-bp isoform was present in cells maintained in a hypoxic environment. Four of the 11 translated exons predicted to code for the 1305-bp product were spliced out of the 936-bp transcript. The 1305-bp product enhanced expression from the VEGF promoter 4-fold greater than background, whereas the 936-bp and the 447-bp isoforms enhanced expression 3× and 12×, respectively. Analysis with deletion promoter constructs determined that all isoforms required the presence of Sp1 elements for efficient activation and that the hypoxia response element (HRE) was not essential for enhancement. Transcripts for novel *RTEF-1* isoforms were also identified in neural retinal tissue of mice. Different murine-specific isoforms were present at different stages of postnatal development.

Conclusions. Novel *RTEF-1* transcripts are present within human ocular vascular endothelial cells and mouse neural retina during normal and ROP development, and alternatively spliced products are produced under hyperoxic and hypoxic conditions. Alternative spliced variants of human RTEF-1 transcripts are able to potentiate expression from the *VEGF* 5′ proximal promoter region. (*Invest Ophthalmol Vis Sci.* 2007;48:3775–3782) DOI: 10.1167/iovs.06-1172

Transcriptional enhancer factor 1-related (*RTEF-1*) gene is a member of the TEA DNA binding domain gene family. The TEA DNA-binding domain gene family is highly conserved from *Aspergillus nidulans*, yeast, *Drosophila*, and mice to humans. The TEA DNA-binding family of proteins can be involved in the activation and repression of multiple genes, and their particular function can be modified by association with other proteins.[1] Expression of specific members of these genes has been identified in various mammalian tissues, including heart, skeletal muscle, pancreas, placenta, brain, and lung.[2–4] Isoforms arising from alternative splicing of mRNA from a single gene, for transcriptional enhancer factor-1 (*TEF-1*), have been identified within a single tissue such as the pancreas.[5,6] The expression profile of these genes within the mammalian eye has not been reported.

Transcripts of the *RTEF-1* gene were first identified in chicken tissue and were demonstrated to be enriched in cardiac and skeletal muscle.[4] Chicken RTEF-1 binds to the myocyte-specific CAT (M-CAT) *cis* DNA elements, regulates the expression of muscle-specific genes, and requires muscle-specific cofactors for full transcriptional activation. Random screening of 2166 clones from a human colorectal cancer cDNA library identified a partial cDNA RTEF-1 sequence that led to the isolation of a full-length human homolog of the avian RTEF-1 from a heart cDNA library.[2,7] Northern blot analysis of human tissue indicated the highest levels of expression in skeletal muscle and pancreas, with lower levels in the heart, kidney, and placenta, whereas the message was not detected in the liver, lung, or brain.[2] Northern blot analysis of the mouse homolog of RTEF-1 indicates a different tissue expression pattern when compared with that in humans. Adult mouse lung tissue expressed the highest level, with very low levels in kidney, heart, and skeletal muscle and undetectable amounts in liver, thymus, spleen, and brain, whereas RTEF-1 message was abundant in mouse embryonic skeletal muscle.[8] An alternatively spliced mouse isoform of RTEF-1 that lacks exon 5 compared with the full-length gene has been identified in mouse skeletal muscle cells.[8]

Recently, the full-length RTEF-1 protein has been identified not only to bind to the VEGF promoter but also to upregulate the expression of VEGF under hypoxic conditions in bovine aortic endothelial cells (BAECs).[9] Microarray analysis revealed

From the Casey Eye Institute, Oregon Health and Science University, Portland, Oregon.

Supported by the Clayton Foundation for Research, Research to Prevent Blindness, the Knights Templar Eye Foundation, the Stan and Madelle Rosenfeld Family Trust, and National Institutes of Health Grants EY010572, EY003279, EY011548, and EY008247. JTR is a senior scholar supported by RPB.

Submitted for publication September 29, 2006; revised February 26, 2007; accepted June 11, 2007.

Disclosure: **B. Appukuttan**, None; **T.J. McFarland**, None; **M.H. Davies**, None; **L.-o. Atchaneeyasakul**, None; **Y. Zhang**, None; **B. Babra**, None; **Y. Pan**, None; **J.T. Rosenbaum**, None; **T. Acott**, None; **M.R. Powers**, None; **J.T. Stout**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: J. Timothy Stout, Casey Eye Institute, Oregon Health and Science University, 3375 SW Terwilliger Boulevard, Portland, OR 97239; stoutt@ohsu.edu.

that RTEF-1 expression was upregulated by 3-fold in BAECs under hypoxic conditions. Surprisingly, RTEF-1 mediated VEGF gene activation through interaction with Sp1 elements within the VEGF promoter and not with M-CAT motifs. In addition, RTEF-mediated expression of VEGF is achieved independently of the hypoxia-inducible factor (HIF-1) and hypoxia responsive element (HRE) pathways of activation.[9]

Given that VEGF plays a key role in the development of various ocular neovascular diseases, it is plausible that RTEF-1 may also play a role in the pathogenesis of proliferative retinopathies. We examined human retinal vascular endothelial cells for the presence of RTEF-1 mRNA isoforms and tested whether these products could also affect transcription directed by sequences upstream of the VEGF gene. We also sought to determine whether RTEF-1 transcripts are present in vivo in retinal tissue from a mouse model of retinopathy of prematurity (ROP).

METHODS

Primary Ocular Vascular Endothelial Cell Isolation and Culture

All use of human cells and tissue was in accordance with approved institutional review board protocols. Primary cultures of endothelial cells isolated from human retina were established using established protocols[10,11] and were used as a source for mRNA. Human cadaver eyes were obtained from anonymous donors (Lions Eye Bank, Portland, OR) within 24 hours of death. Donors had no history of cardiovascular or ocular disease and ranged in age from 16 to 42. Briefly, these retinal and iris tissues were aseptically dissected and separated from donor eyes and were digested in 0.2% collagenase (Sigma Chemical Co., St. Louis, MO). Endothelial cells (ECs) were isolated from other cell types with the use of mouse monoclonal anti-human CD31 antibody-coated magnetic beads (Dynal Biotech, Inc., Lake Success, NY). ECs were cultured in complete MCDB-131 medium (Clonetics/BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum and antibiotics. Cells were used at passages 2 to 5. After two rounds of magnetic bead separation, the EC cultures were greater than 99.5% pure, as evaluated by morphologic criteria, expression of CD31 and von Willebrand factor, and uptake of acetylated low-density lipoprotein.[11]

Induction of Hypoxia

Retinal endothelial cells were cultured to 80% confluence in 60-mm-diameter culture dishes and then were placed in an air-tight chamber (Modulator Incubator Chamber; Billups-Rothenberg, Del Mar, CA). A gas mixture consisting of 1% O_2, 5% CO_2, and the remainder N_2 was flushed through the chamber for exactly 5 minutes, whereupon the chamber was sealed and placed in a humidified 37°C incubator. After 8 hours, the chamber was flushed again for 5 minutes with the hypoxic gas mixture, sealed, incubated for 8 more hours, flushed again, and incubated for another 8 hours, at which point total RNA was isolated.

Total RNA Extraction and RT-PCR

Total RNA was isolated with the use of an RNA purification kit (RNAqueous; Ambion Inc., Austin, TX) according to manufacturer's protocol, and 50 ng of this RNA was used with oligo-dT primer first-strand synthesis (SuperScript II; Stratagene, La Jolla, CA). Human primers F1 (5′-ttggagggcacggccggca-3′) and R1 (5′-tcattctttcaccagcctgta-3′), designed from the published human RTEF-1 sequence (accession no. U63824), were used for second-strand PCR amplification under standard conditions. Mouse primers F2 (5′-cctgggaccggtggaac-3′) and R2 (5′-tgcaggatggtgaagttc-3′), designed from published mouse sequence (accession no. D87965), were used for cDNA amplification. Amplified products were electrophoresed and visualized in a 1.5% agarose gel and subsequently purified from the gel (QIAquick Gel Extraction; Qiagen, Valencia, CA) for standard dideoxynucleotide sequencing on an automated sequencer (ABI 310; Applied Biosystems, Foster City, CA).

Reporter Gene Analysis

Full-length RTEF-1 isoforms were directionally cloned into the pcDNA 3.1 expression plasmid (Invitrogen, Carlsbad, CA). The predicted TTG start was converted to ATG within the forward primer sequence. Human VEGF 5′ proximal promoter fragment of 1136 bp (F1-R3) containing 54 bp of 5′UTR and 1082 bp upstream of the transcription start site was directionally cloned 5′ to the secretable alkaline phosphatase (*SEAP*) gene within the pSEAP reporter plasmid (Clontech, Mountain View, CA). A truncated human VEGF 5′ proximal promoter fragment of 634 bp (F2-R3), containing 54 bp of 5′UTR and 580 bp upstream of the transcription start site, was also directionally cloned into the pSEAP plasmid. Promoter fragments with deletions were constructed by amplification of the 5′ end of the promoter and the 3′ end of the promoter and subsequent ligation of the amplified products. The ligated products lacking the region of interest were then amplified and directionally cloned into the promoterless pSEAP vector. All constructs were sequenced on both strands for verification before transfection studies.

Transfection Assays

Transfection was performed using the Amaxa nucleofection device and reagents (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's standard protocol. Briefly, 293T cells were cultured in 10% DMEM until they were 80% confluent, and then they were trypsinized and collected. Half a million cells were used for each nucleofection. Half a million cells were resuspended in 100 μL solution (Nucleofect; Amaxa) and 5 μL (containing 2 μg) total plasmid DNA and electroporated (program A023 on Nucleofection Device; Amaxa) and then were immediately resuspended in 1 mL prewarmed media and seeded into a single well of a six-well plate. Cells were allowed to recover for 16 to 18 hours, and the media were carefully removed and replaced with exactly 500 μL fresh media. After exactly 24 hours of incubation, 150 μL media were carefully removed and 25 μL of this was either assayed immediately or stored at −20°C for future SEAP analysis. Three separate 25-μL media aliquots were used for SEAP analysis according to manufacturer's protocol (BD Biosciences, San Jose, CA), and the SEAP values for all three readings were averaged for comparison with triplicate repeat experiments.

Each cotransfection was repeated at least three times in a single experiment, and each experiment was repeated independently two more times with separate plasmid preparations ($n = 9$–12). Results of one representative experiment performed in triplicate are presented. Statistical analysis was performed using a Student's t-test (two-tailed) to compare the three or four samples in a single experiment. Bonferroni correction for multiple testing was applied, and $P < 0.01$ was considered significant.

For each cotransfection assay (when two plasmids were transfected together in the same tube), the copy number of each plasmid was adjusted to be equivalent to the copy number of the largest plasmid used. The pSEAP vector without a promoter and the pcDNA 3.1 expression plasmid with no insert served as negative controls. For each nucleofection experiment, two separate positive control plasmids, a SV40 promoter pSEAP plasmid and a pGFPmax vector, were transfected simultaneously to ensure efficient and equal transfection efficiencies. The pSEAP plasmid with an SV40 promoter served as a positive control for subsequent SEAP protein analysis. The pGFPmax vector also served as positive control for transfection for each batch of cells, allowing visual confirmation of consistent transfection efficiency. Nucleofection consistently gave 80% to 90% transfection efficiency in 293T cells in all experiments.

Mouse Model of Retinopathy of Prematurity

The procedure for the development of ROP in mice has been described previously.[12,13] Briefly, C57BL/6 (B6) mice were purchased (Simonsen

Laboratories, Gilroy, CA) and bred at the Oregon Health and Science University animal care facility in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To induce retinopathy, postnatal day (P)7 mice, with nursing mothers, were exposed to hyperoxic conditions (75% oxygen) for 5 days. At P12 the hyperoxic exposed mice were recovered in room air. Control litters were maintained at normal room air conditions. Pups exposed to high oxygen and control pups exposed to normal air were humanely euthanatized on P8, a hyperoxic stage, and P17, a relatively hypoxic stage corresponding to peak time of neovascularization. Both eyes were immediately enucleated from each mouse and were dissected for careful removal of the neural retina. Retinal tissues from four eyes, two from each of two littermates at the same stage of development, were pooled for isolation of total RNA.

Results

Novel Isoforms of RTEF-1 Exist within Hypoxic and Normal Ocular Vascular Endothelial Cells

Amplification from cDNA prepared from primary cultures of human retinal vascular endothelial cells (PRVECs) and iris VECs (PIVECs), using the F1 and R1 primer pair, gave products of approximately 1300 bp and 900 bp (Fig. 1). Identical primer pair amplification from cDNA isolated from PRVECs that had been cultured under hypoxic conditions for 24 hours, before isolation of mRNA, gave an additional product of approximately 450 bp (Fig. 1).

Sequencing analysis revealed that the largest product was identical with the full-length 1305-bp *RTEF-1* gene spanning from the start to the stop codon, whereas 900 bp and 450 bp were 936-bp and 447-bp alternate spliced transcripts of the 1305-bp product. The following description of codons will be numbered according to the sequence in the 1305-bp transcript, consisting of 435 codons, with the protein initiating codon numbered 1 and the stop codon numbered 435. Exons 5 to 8, four of the eleven exons that are predicted to code for the protein portion of the 1305-bp transcript, are spliced out of the 936-bp version (Fig. 2). Exon 5 was absent from the 447-bp isoform, and an unusual in-frame splice event occurs in the middle of exon 7, which splices out from Gln-83 in exon 7 to codon Gln-425 within exon 12 (Fig. 2).

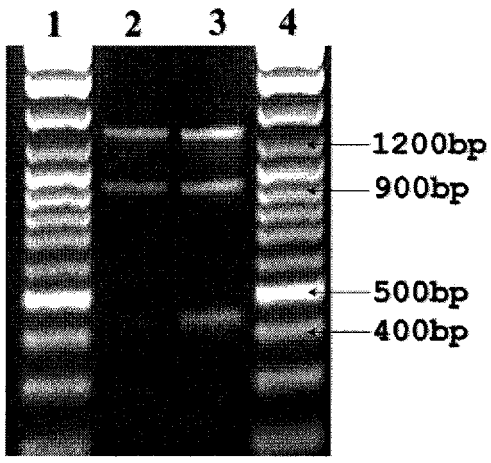


FIGURE 1. Agarose gel electrophoresis showing RT-PCR of RTEF-1 from cDNA prepared from primary cultures of human RVECs. *Lanes 1* and *4*, DNA ladder; *lane 2*, cDNA prepared from RVEC under normoxic conditions gave 2 products (approximately 1300 and 900 bp); *lane 3*, cDNA prepared from RVECs under hypoxic conditions gave 3 products (approximately 1300, 900, and 450 bp).

The 1305-bp product shows identity to the transcriptional enhancer factor-1 related (*RTEF-1*) gene originally identified in human heart, skeletal muscle, pancreas, and lung tissue.[2] Two other RTEF isoforms—variant 2 (accession no. NM_201441), which lacks exon 5 from Asp-119 to Gly-161, and variant 3 (accession no. NM_201443), which makes use of a downstream protein initiation site at Met-130—have been reported. The 936-bp and 447-bp isoforms identified within human ocular vascular cells have not been identified in any other human tissue to date.

The predicted protein sequence for the 936-bp and 447-bp isoforms contain the 72-amino acid TEA domain (Asp-38 to Lys-109), which contains three predicted α-helices and a putative nuclear localization signal (Leu-105 to Lys-109). However within the C-terminal domain, a proline rich-domain (Pro-189 to Pro-213) spanning the last six amino acids of exon 7 and the first 19 residues of exon 8 are missing from the 447-bp isoform (Fig. 2). In addition, two domains of STY (Ser-253 to Ser-271 and Ser-311 to Ser-336), a region laden with hydroxylated residues such as serine, threonine, and tyrosine—one located within exon 9 and the other within exon 10—are also lacking in the 447-bp isoform (Fig. 2).

Novel Isoforms of RTEF-1 Are Able to Upregulate Expression from the VEGF Promoter

It has been shown that the 1305-bp isoform acts as a transcriptional stimulator of VEGF, in bovine aortic endothelial cells, by binding to an Sp1 site.[9] We investigated whether the new isoforms were also capable of stimulating expression from the human VEGF promoter. The 5′ proximal promoter of the human VEGF gene, 54 bp of the 5′UTR and 1082 bp upstream of the transcription initiation site (Fig. 3A), was cloned into a pSEAP reporter plasmid, and the three RTEF isoforms were cloned into a pcDNA expression vector. Because of the difficulties in nucleotransfection of plasmid DNA into primary cultures of ocular vascular endothelial cells, 293T cells were used as a substitute cell line for transfection studies. Cotransfection of the VEGF promoter-reporter plasmid with any of the three isoforms indicated that all isoforms upregulated expression of the reporter from the VEGF promoter (Fig. 4). The full-length 1305-bp RTEF-1 product and the 936-bp isoform enhanced expression between 3-fold and 4-fold, significantly higher than background ($P < 0.005$), and no difference was observed between these two isoforms ($P > 0.01$) after correcting for multiple testing. The 447-bp isoform stimulated expression approximately 10- to 15-fold (average, 12×) above background expression ($P < 0.001$). Each cotransfection experiment was repeated in triplicate on three separate occasions with the same results.

Sp1 Elements Are Required for VEGF Promoter Activity but May Not Be Essential for RTEF-1 Enhancer Activity

Earlier studies demonstrated that the full-length RTEF-1 isoform binds to and requires an Sp1 element for augmentation of VEGF promoter activity. In a previous study, mutation of this Sp1 site situated at -97 to -89 bp, resulted in a loss of RTEF-1 enhancer activity.[9] In the same study, three other Sp1 sites within the same region, -86 to -58 bp, were found not to be essential for RTEF-1 enhancer activity. To test whether the new RTEF-1 isoforms required Sp1 sites for enhancer activity, the VEGF promoter, with all four Sp1 sites deleted (Fig. 3B), from -113 bp to -58 bp, was cloned into a pSEAP vector and was cotransfected with each isoform. Comparison of background reporter gene expression from the full-length and the Sp1-negative ($Sp1^-$) VEGF promoter indicated that loss of Sp1 elements resulted in a dramatic 30-fold decrease in reporter

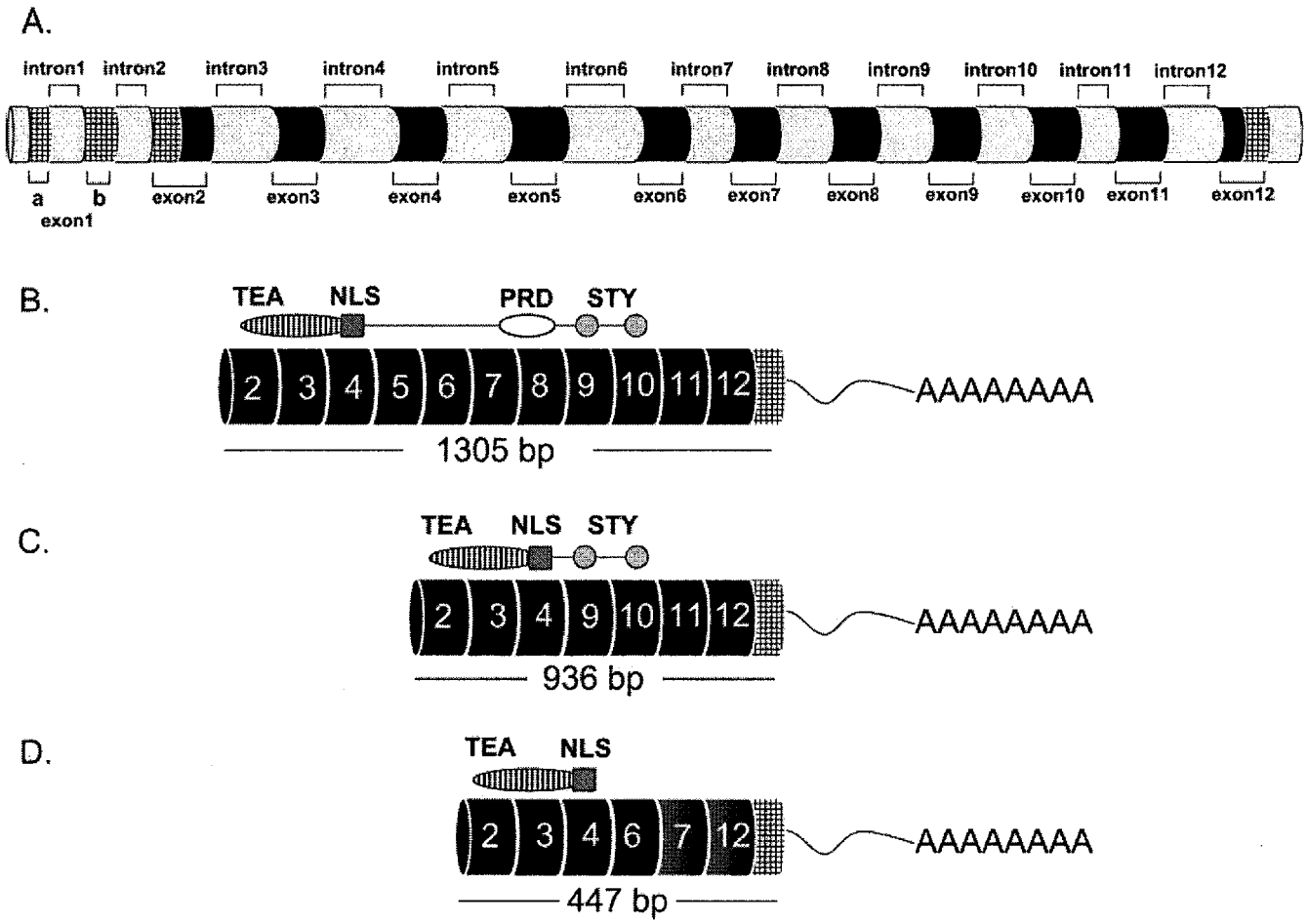


FIGURE 2. Schematic of the genomic structure of RTEF-1 and the three isoforms isolated from human retinal endothelial cells. (**A**) *Hatched* areas indicate 5′ and 3′ untranslated exons, and *solid black* areas indicate protein coding exonic regions. (**B**) Full-length RTEF-1 (1305 bp) from the initiating start codon within exon 2 to the stop codon in exon 12 contains the TEA DNA-binding domain (TEA, *oval with vertical stripes*), a putative nuclear localization signal (NLS), a proline rich domain (PRD) and 2 serine-threonine-tyrosine (STY) domains. The *square* shows the position of the NLS, the *open oval* shows the PRD, and the *circles* show the STY domains. (**C**) The 936-bp isoform lacks exons 5 to 8 and is missing the PRD. (**D**) The 447-bp isoform lacks the PRD and the STY domains and is spliced in such a way as to contain only the 5′ end of exon 7 and the 3′ end of exon 12.

expression (Fig. 5A), suggesting that at least one of the four Sp1 elements within the proximal promoter is essential for expression. The RTEF-1 isoforms could not enhance expression from the promoter without the Sp1 elements to levels observed with the F1-R3 promoter (Fig. 5A). However, a closer look at the effect of each isoform on the Sp1-negative promoter (Fig. 5B) relative to the background expression observed from the control indicated that a similar trend of enhancement was observed, representing 3-fold, 4-fold, and 12-fold enhancement above background control expression for the 1305-bp, 936-bp, and 450-bp isoforms, respectively. Thus, it would appear that the level of enhancement relative to the background control afforded by each isoform is comparable regardless of whether Sp1 elements are present within the VEGF promoter.

Novel Isoforms of RTEF-1 Do Not Require the HRE

The hypoxia response element (HRE) sequence, situated between -985 and -939 bp with a core sequence (-975 and -968 bp) within this region, is essential for the binding of hypoxia-inducible factor (HIF)-1α, which is responsible for the enhancement of VEGF expression under conditions of low oxygen.[14] We investigated whether the RTEF isoforms, specifically the 450-bp isoform, observed under hypoxic conditions and identified in this study, require the presence of the HRE within the VEGF promoter for enhancement activity. A truncated version of the 5′ proximal promoter region of the human VEGF gene, from -580 bp spanning 54 bp of 5′UTR, was cloned into a pSEAP plasmid (F2-R3; Fig. 3C). Cotransfection of this promoter with each RTEF-1 isoform implies that all isoforms are able to stimulate expression of the VEGF promoter lacking the HRE region above background expression observed from the control (Fig. 6). Only the 936-bp and 447-bp isoforms were thought to show a significant difference compared with the no insert control ($P < 0.0001$) and enhanced between 2- to 3-fold and 8- to 12-fold above the control. Again, the 447-bp isoform gave the most robust stimulation compared with the other isoforms. The full-length 1305-bp product only gave a slight enhancement above background (less than 2-fold) and might have required the HRE or other nearby sequences upstream of -580 bp for efficient enhancement, as observed with the F1-R3 promoter.

Novel RTEF-1 Isoforms Are Present within Murine Retinal Tissue

Amplification from cDNA prepared from neural retinal tissue isolated from normoxic (control) and hyperoxic exposed mice

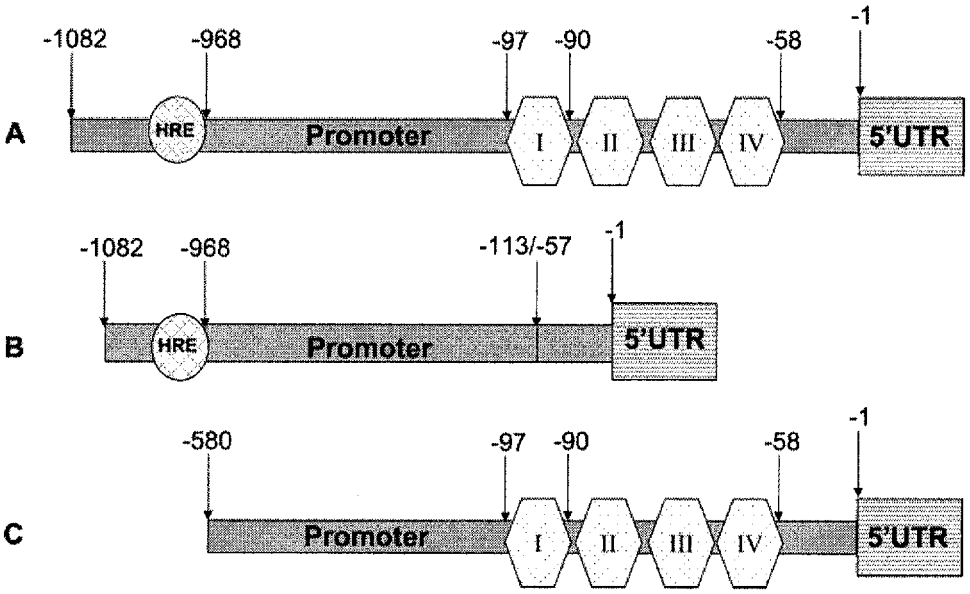


FIGURE 3. Schematic of the human VEGF promoter cloned for expression analysis (not to scale). (A) Native promoter (F1-R3) containing 54 bp of the 5′ untranslated region (5′UTR) 1082 bp upstream of the transcription initiation site (-1 bp) with the position of the HRE and 4 Sp1 sites (I, II, III, IV). (B) Sp1-deleted promoter (F1-R3 Sp1⁻), which lacks a 56-bp region between -57 and -113-bp and contains four Sp1 sites. (C) F2-R3 VEGF promoter construct lacks the HRE motif and starts at -580-bp.

at P8 and P17, using the F2 and R2 primer pair, demonstrated the presence of the 1305-bp RTEF-1 isoform. This was confirmed by sequence analysis (Fig. 7). Interestingly, additional amplified products were present, a 920-bp fragment in the P8 control, an 850-bp fragment in the P17 control, and 1100-bp and 610-bp bands in P17 hyperoxia exposed samples (Fig. 7). Sequencing analysis showed that all extra fragments were alternatively spliced RTEF-1 isoforms (data not shown). The mouse RTEF-1 protein coding exonic structure was identical to the human RTEF-1 exon architecture; thus, Figure 2B can be used as a reference. The 1100-bp isoform, which lacks exon 5, has been identified in craniofacial tissue (not including brain or eye tissue) from 12.5-day-old mouse embryos.[3] The other three isoforms are novel and unique to mouse. The 920-bp isoform identified in P8 normoxic retina lacks exons 5, 7, and 8, whereas the 850-bp isoform within the P17 normoxic retina lacks exons 5, 8, and 9. The 920-bp isoform lacks the entire PRD region (contained within exons 7 and 8) and retains the 2 STY domains within exons 9 and 10, whereas the 850-bp isoform lacks exon 8. Thus, the 850-bp isoform has only 7 of the 13 proline residues contained within the PRD and retains only one of the STY domains, which is in exon 9. The 610-bp P17 ROP-specific isoform is spliced from within exon 2, just before the start of the TEA domain, and links again to exon 8. Thus, the 610-bp isoform lacks most of exon 2 and all of exons 3 to 7, which means the entire TEA domain, the nuclear localization signal (NLS), and five proline residues of the PRD are missing.

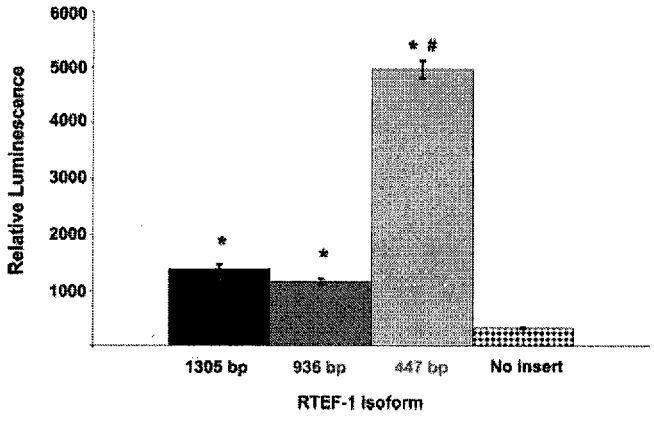


FIGURE 4. All RTEF-1 isoforms enhance expression from the full-length VEGF (F1-R3) promoter. The 1305-, 936-, or 447-bp isoform or a pcDNA no insert control was cotransfected with a human VEGF (F1-R3) promoter pSEAP reporter construct ($n = 12$). One representative triplicate experiment is shown. *Bars* indicate reporter protein levels driven from the F1-R3 promoter when cells were cotransfected with pcDNA containing no insert or one of the RTEF-1 isoforms. All products showed significant differences (*) when compared with the no insert control ($P < 0.005$). No difference was observed between the 1305- and 936-bp isoforms. The 447-bp isoform was significantly different (#) from either of the two larger isoforms ($P < 0.001$).

DISCUSSION

In this study we examined whether RTEF-1 is expressed within the human ocular vascular endothelial cells. We demonstrated that RTEF-1 and other previously undescribed alternatively spliced isoforms are expressed within human ocular vascular endothelial cells. The 447-bp isoform is present under hypoxic conditions. We showed that all isoforms are capable of enhancing expression from the human VEGF 5′ proximal promoter and that 447-bp "hypoxic" isoform exhibits the most potent effect of the three isoforms tested.

To be able to design useful therapies, we must first hope for an understanding of the molecular events that lead to the onset and progression of disease. It is well established that VEGF plays an important role in the development and severity of ROP and other ocular neovascular diseases.[15–18] Thus, understanding how VEGF gene expression is regulated and which factors are involved in this process will allow us to better understand the etiology of neovascular disease and to develop new targets for therapeutic intervention. The RTEF-1 protein is able to bind to the VEGF promoter and to upregulate expression of the VEGF gene under hypoxic conditions in BAECs.[9] We investigated whether RTEF-1 mRNA is present within human retinal endothelial cells in culture under normoxic and hypoxic conditions.

The discovery of two new isoforms, one of which is present only under hypoxic conditions in human retinal vasculature, suggests that tissue-specific and possibly disease-specific isoforms exist for RTEF-1. Another member of the TEA domain family of genes, TEF-1, is also alternatively spliced into multiple

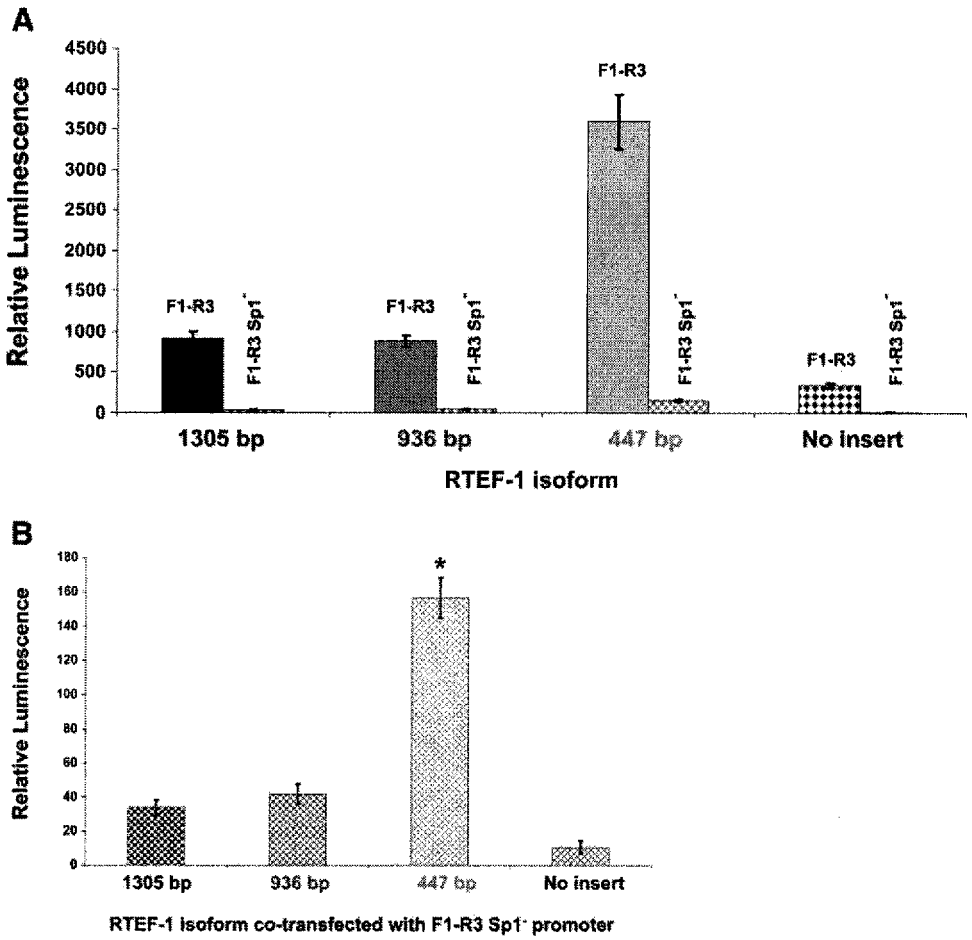


FIGURE 5. (A) All RTEF-1 isoforms require an Sp1-rich region within the VEGF promoter for efficient enhancement of expression. The 1305-, 936-, or 447-bp isoform or a pcDNA no insert control was cotransfected separately with a pSEAP reporter construct containing a human VEGF (F1-R3) promoter or a VEGF promoter lacking a 56-bp region containing four Sp1 sites (F1-R3 Sp1⁻). One representative triplicate experiment is shown ($n = 12$). *Solid columns* show relative reporter protein levels driven from the full-length promoter (F1-R3), and *hatched columns* juxtaposed immediately to the right of the *solid columns* indicate relative reporter levels from the promoter lacking the Sp1 sites (F1-R3 Sp1⁻) when cells were cotransfected with the equivalent isoform. All isoforms showed a significant reduction in enhancement from the F1-R3 Sp1- promoter compared with the full-length F1-R3 promoter ($P < 0.004$). Thus, all RTEF-1 isoforms require an Sp1-rich region for full-strength enhancement. (**B**) Comparison of the effect of each RTEF-1 isoform induced enhancement from the human VEGF promoter lacking Sp1 sites (F1-R3 Sp1⁻). The 1305, 936- or 447-bp isoform or a pcDNA no insert control was cotransfected with an F1-R3 Sp1⁻ promoter pSEAP reporter construct ($n = 12$). Although overall enhancement is drastically reduced relative to the full-length F1-R3 VEGF promoter (Fig. 5A) the trend of enhancement by each isoform relative to the background expression, as indicated by the no insert control, is similar. The 1305-bp ($P = 0.027$) and 936-bp ($P = 0.035$) isoforms gave approximately 3- to 4-fold higher enhancement above background, whereas the 447-bp ($P = 0.009$) isoform increased expression by approximately 12-fold. In this study, the 447-bp isoform was the only isoform considered to give a significant enhancement difference (*) when compared with the no insert control ($P < 0.01$).

isoforms. These isoforms differ between normal and cancerous pancreatic cells.[5] Thus, it is possible that the 447-bp RTEF-1 isoform is expressed under conditions of disease. Whether this isoform is specific to retinal vascular tissue or is also present within other ocular cells or other human tissue remains to be determined. It would be of interest to determine whether RTEF-1 isoforms play a role in the etiology of human neovascular eye disease. To show that RTEF-1 exists in vivo within the mammalian retina, we looked for and identified transcripts (Fig. 7) of alternatively spliced RTEF-1 isoforms during mouse neural retina development and ROP disease development (Appukuttan B, et al. *IOVS* 2005;46:ARVO E-Abstract 3117). Not only are some of the mouse isoforms novel, they are unique to mouse tissue. In addition, specific isoforms are only present during particular stages of disease or normal development (Fig. 7). It is possible that retinal-specific isoforms exist that are unique to mice and humans and, though species specific, may have similar roles during disease and normal retinal development.

Human RTEF-1 was originally cloned from a heart cDNA library and was shown, by Northern blot analysis, to be abundantly expressed in human skeletal muscle and pancreas, less expressed in heart and kidney, and absent from brain, liver, and lung.[2] Subsequently, RTEF-1 has been shown to regulate gene expression through myocyte-specific CAT elements and may play a role in α1-adrenergic-induced hypertrophy of cardiac myocytes.[19,20] Mouse RTEF-1 also binds to M-CAT elements and is involved in skeletal muscle–specific expression and embryogenesis.[8,21] In a previous report, RTEF-1–mediated VEGF gene activation required interaction with an Sp1 element within the VEGF promoter and not M-CAT motifs within BAECs.[9] Mutation of the Sp1 site, situated at -97 to -89 bp, resulted in the abolishment of RTEF-1 enhancer activity, resulting in reporter gene expression equivalent to background expression levels. We observed a sharp decrease in overall expression from the VEGF promoter when Sp1 sites were deleted (Fig. 5A). Others have also shown that mutation of these Sp1 sites results in a natural reduction in VEGF promoter activity compared with the native promoter. Interestingly, enhancer activity is completely abolished for certain factors, such as platelet-derived growth factor, that normally requires these Sp1 sites for activity.[22] In contrast, all RTEF-1 isoforms still mediated some level of enhancement from the VEGF Sp1-negative promoter compared with the control, even though the SP1 sites were deleted (Fig. 5B). Although not conclusive, it is possible that these isoforms use sites other than Sp1 sites for enhancement. It is also possible that RTEF-1 regulates expression through an Sp1 site in BAECs but that RTEF-1 and isoforms modify expression in 293T cells through an alternative mechanism. Bovine endothelial cells may contain cell-specific cofactors necessary for RTEF-1/Sp1–mediated expression that are lacking in 293T cells. It would be of interest to determine whether RTEF-1 isoforms have the same effect on the VEGF promoter in various cell types.

The 447-bp RTEF-1 isoform isolated in this study was the most powerful stimulator of expression. This isoform was isolated from hypoxic cells, and it is well documented that VEGF mRNA levels are increased in human and rodent eyes under hypoxic disease conditions.[18,23–25] Thus, this isoform is still able to stimulate enhanced expression of a VEGF promoter that lacks an HRE region, the presence of which is normally crucial for VEGF expression induction under hypoxic conditions (Fig. 6). How an alternatively spliced isoform that codes for a protein lacking 65% of the "normal" full-length content is capable of augmenting function remains unclear. The 447-bp isoform contains the complete 72-amino acid TEA domain with nuclear localization signal, as do the other two larger isoforms. The 447-bp and the 936-bp isoforms lack exons 5 and 8, implying that the moieties responsible for the differences in enhancement lie elsewhere. A proline-rich domain (PRD), an activation domain common to other TEA domain proteins, exists within RTEF-1 between amino acids Pro-189 and Pro-213, encoded mainly by exons 7 and 8. This 25-amino acid stretch contains 12 proline residues. The PRD in TEF-1 is crucial for the activation of gene expression but is not required for the function of RTEF-1 in mouse skeletal muscle or human cervical carcinoma (HeLa) cells.[8] Thus, although the 936-bp isoform lacks the PRD, this is unlikely to be the cause of the variation given that the 1305-bp isoform containing the PRD exhibits levels of enhancement similar to the latter. Deletion and chimeric constructs of RTEF-1 and TEF-1 imply that the α1-adrenergic transactivation function of RTEF-1 is located within the conserved 200 amino acids at the carboxyl-terminal end.[20] This 200-amino acid region spans exons 9 to 12, and the 447-bp isoform contains only nine amino acids of exon 12 (Fig. 2). Of the three human RTEF-1 isoforms isolated, and considering that the 447-bp isoform is the most potent enhancer of the VEGF promoter, it seems that a combination of the loss of the carboxyl-terminal end and the PRD is somehow responsible for the amplification of activation.

Another plausible explanation for the function of the 447-bp isoform is the lack of two STY-rich domains. The STY-rich domains are regions rich in serine, threonine, and tyrosine residues, which are putative activation domains.[26–28] The STY-rich domains within RTEF-1 are situated between amino acids Ser-253 and Ser-271, within exon 9 and between Ser-311 and Ser-336 within exon 10.[8] The 447-bp isoform, unlike the other two isoforms, is missing exons 9 and 10. Serine, threonine, and tyrosine residues all have the potential to be phosphorylated, which can profoundly affect the biological activity of a protein. It is possible that the loss of potential phosphorylation sites within RTEF-1 may be responsible for the enhanced ability of the shortest isoform to stimulate expression. The STY-rich domain within exon 10 conforms to a consensus MAPK site.[29] Mutation of this MAPK site results in the loss of potentiation of an α1-adrenergic response in cardiac myocytes,[20] and the deletion of only the STY-rich domain in exon 9 results in loss of transactivation within mouse skeletal muscle and HeLa cells.[8] Further investigation into the new isoforms is required to discern the molecular mechanisms of expression enhancement.

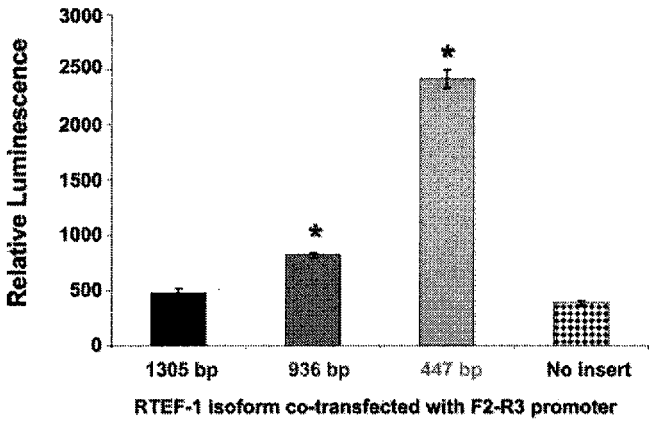


FIGURE 6. RTEF-1 isoforms do not require the presence of the HRE sequence for the enhancement of expression from the VEGF promoter. *Columns* indicate relative luminescence of reporter protein levels driven from the F2-R3 promoter when cells were cotransfected with pcDNA containing no insert or one of the RTEF-1 isoforms. Results of one experiment performed in triplicate are shown ($n = 9$). *Asterisk:* the 936-bp and 447-bp isoforms showed a significant difference compared with the no insert control ($P < 0.0003$), whereas the 1305-bp product showed no difference ($P = 0.027$; $P > 0.01$). A significant difference was also observed between the 936-bp isoform and the shortest isoform ($P < 0.0001$).

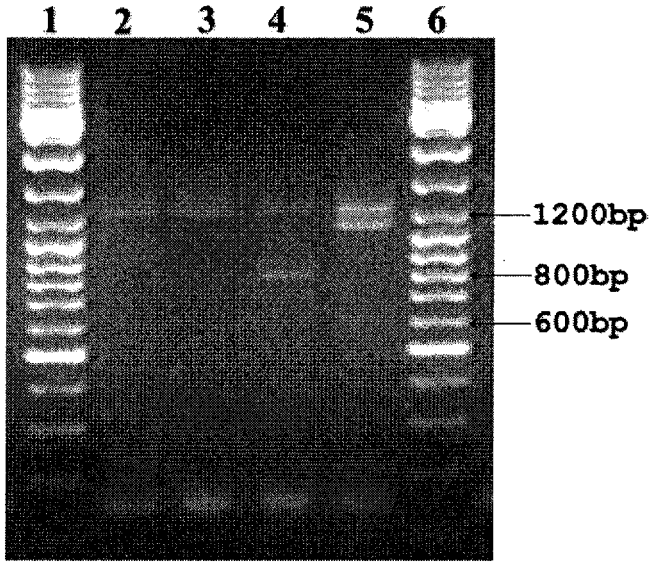


FIGURE 7. Agarose gel electrophoresis showing RT-PCR of RTEF-1 from cDNA prepared from mouse neural retina under normal development and conditions of ROP. *Lanes 1* and *6*, DNA ladder; *lane 2*, cDNA prepared from P8 mice under normoxic conditions gave 2 products (approximately 1300 and 920 bp); *lane 3*, cDNA prepared from P8 ROP mice gave 1 product (approximately 1300 bp); *lane 4*, cDNA prepared from P17 mice under normoxic conditions gave 2 products (approximately 1300 and 800 bp); *lane 5*, cDNA prepared from P17 ROP mice gave 3 products (approximately 1300 bp, 1100 bp, and 610 bp).

In summary, the *RTEF-1* gene is expressed within human ocular-derived vascular cells and mouse retina. Novel murine-specific isoforms have been identified that may play a role in normal retinal development or in the development of retinal disease. Alternatively spliced novel human RTEF-1 isoforms have been identified that confer different transcriptional enhancer activity of the human VEGF promoter. A novel 447-bp isoform present within hypoxic retinal endothelial cells shows greater activation of expression from the VEGF promoter than other human isoforms. Developing therapies that target the 447-bp product for the treatment of ocular neovascular disease may prove beneficial.

Acknowledgments

The authors thank Genevieve Long for editorial comments and Beth Wilmot for advice and critical discussion.

References

1. Kaneko KJ, DePamphilis ML. Regulation of gene expression at the beginning of mammalian development and the TEAD family of transcription factors. *Dev Genet*. 1998;22:43–55.
2. Stewart AF, Richard CW 3rd, Suzow J, et al. Cloning of human RTEF-1, a transcriptional enhancer factor-1-related gene preferentially expressed in skeletal muscle: evidence for an ancient multigene family. *Genomics*. 1996;37:68–76.
3. Yasunami M, Suzuki K, Ohkubo H. A novel family of TEA domain-containing transcription factors with distinct spatiotemporal expression patterns. *Biochem Biophys Res Commun*. 1996;228:365–370.
4. Farrance IK, Ordahl CP. The role of transcription enhancer factor-1 (TEF-1) related proteins in the formation of M-CAT binding complexes in muscle and non-muscle tissues. *J Biol Chem*. 1996;271:8266–8274.
5. Zuzarte PC, Farrance IK, Simpson PC, et al. Tumor cell splice variants of the transcription factor TEF-1 induced by SV40 T-antigen transformation. *Biochim Biophys Acta*. 2000;1517:82–90.
6. Jiang SW, Trujillo MA, Sakagashira M, et al. Novel human TEF-1 isoforms exhibit altered DNA binding and functional properties. *Biochemistry*. 2000;39:3505–3513.
7. Frigerio JM, Berthezene P, Garrido P, et al. Analysis of 2166 clones from a human colorectal cancer cDNA library by partial sequencing. *Hum Mol Genet*. 1995;4:37–43.
8. Yockey CE, Smith G, Izumo S, et al. cDNA cloning and characterization of murine transcriptional enhancer factor-1-related protein 1, a transcription factor that binds to the M-CAT motif. *J Biol Chem*. 1996;271:3727–3736.
9. Shie JL, Wu G, Wu J, et al. RTEF-1, a novel transcriptional stimulator of vascular endothelial growth factor in hypoxic endothelial cells. *J Biol Chem*. 2004;279:25010–25016.
10. Kanda K, Hayman GT, Silverman MD, et al. Comparison of ICAM-1 and VCAM-1 expression in various human endothelial cell types and smooth muscle cells. *Endothelium*. 1998;6:33–44.
11. Silverman MD, Babra B, Pan Y, et al. Differential E-selectin expression by iris versus retina microvascular endothelial cells cultured from the same individuals. *Microvasc Res*. 2005;70:32–42.
12. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101–111.
13. Davies MH, Eubanks JP, Powers MR. Increased retinal neovascularization in Fas ligand-deficient mice. *Invest Ophthalmol Vis Sci*. 2003;44:3202–3210.
14. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 1996;16:4604–4613.
15. Lashkari K, Hirose T, Yazdany J, et al. Vascular endothelial growth factor and hepatocyte growth factor levels are differentially elevated in patients with advanced retinopathy of prematurity. *Am J Pathol*. 2000;156:1337–1344.
16. Miller JW. Vascular endothelial growth factor and ocular neovascularization. *Am J Pathol*. 1997;151:13–23.
17. Vannay A, Dunai G, Banyasz I, et al. Association of genetic polymorphisms of vascular endothelial growth factor and risk for proliferative retinopathy of prematurity. *Pediatr Res*. 2005;57:396–398.
18. Young TL, Anthony DC, Pierce E, et al. Histopathology and vascular endothelial growth factor in untreated and diode laser-treated retinopathy of prematurity. *J AAPOS*. 1997;1:105–110.
19. Stewart AF, Suzow J, Kubota T, et al. Transcription factor RTEF-1 mediates α1-adrenergic reactivation of the fetal gene program in cardiac myocytes. *Circ Res*. 1998;83:43–49.
20. Ueyama T, Zhu C, Valenzuela YM, et al. Identification of the functional domain in the transcription factor RTEF-1 that mediates alpha 1-adrenergic signaling in hypertrophied cardiac myocytes. *J Biol Chem*. 2000;275:17476–17480.
21. Jacquemin P, Hwang JJ, Martial JA, et al. A novel family of developmentally regulated mammalian transcription factors containing the TEA/ATTS DNA binding domain. *J Biol Chem*. 1996;271:21775–21785.
22. Finkenzeller G, Sparacio A, Technau A, et al. Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression. *Oncogene*. 1997;15:669–676.
23. Pierce EA, Foley ED, Smith LE. Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. *Arch Ophthalmol*. 1996;114:1219–1228.
24. Donahue ML, Phelps DL, Watkins RH, et al. Retinal vascular endothelial growth factor (VEGF) mRNA expression is altered in relation to neovascularization in oxygen induced retinopathy. *Curr Eye Res*. 1996;15:175–184.
25. Pe'er J, Shweiki D, Itin A, et al. Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. *Lab Invest*. 1995;72:638–645.
26. Xiao JH, Davidson I, Matthes H, et al. Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. *Cell*. 1991;65:551–568.
27. Seipel K, Georgiev O, Schaffner W. Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J*. 1992;11:4961–4968.
28. Theill LE, Castrillo JL, Wu D, et al. Dissection of functional domains of the pituitary-specific transcription factor GHF-1. *Nature*. 1989;342:945–948.
29. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem*. 1993;268:14553–14556.

J Gen Virol. 2001 Dec;82(Pt 12):3005-9.

Gene transfer using human polyomavirus BK virus-like particles expressed in insect cells.

Touzé A, Bousarghin L, Ster C, Combita AL, Roingeard P, Coursaget P.

Laboratoire de Virologie Moléculaire, EMI-U 00-10 Protéases et Vectorisation, IFR Transposons et Virus, Faculté des Sciences Pharmaceutiques 'Philippe Maupas', 31 avenue Monge, 37200 Tours, France.

The major structural protein (VP1) of the BK polyomavirus (BKV) was expressed in the recombinant baculovirus expression system. Recombinant BKV VP1 was shown to self-assemble into virus-like particles (VLPs) with a diameter of 45-50 nm. As for other polyomaviruses, BKV VP1 has the capacity to bind to exogenous DNA. Furthermore, the potential of BKV VP1 VLPs was investigated for gene transfer into COS-7 cells using three methods for the formation of pseudo-virions: disassembly/reassembly, osmotic shock and direct interaction between VLPs and reporter plasmid DNA. The latter method was shown to be the most efficient when using linearized plasmid. Gene transfer efficiency with BKV pseudo-virions was of the same order as that observed with human papillomavirus type 16 L1 protein VLPs. In addition, it is demonstrated that cellular entry of BKV pseudo-virions is dependent on cell surface sialic acid.

PMID: 11714977 [PubMed - indexed for MEDLINE]

Intervirology. 2002;45(4-6):308-17.

Generation of recombinant virus-like particles of human and non-human polyomaviruses in yeast Saccharomyces cerevisiae.

Sasnauskas K, Bulavaite A, Hale A, Jin L, Knowles WA, Gedvilaite A, Dargeviciūte A, Bartkeviciūte D, Zvirbliene A, Staniulis J, Brown DW, Ulrich R.

Institutes of Biotechnology, Leeds, UK. sasnausk@ibt.lt

OBJECTIVES: Non-viral methods of gene transfer have been preferred in gene therapy approaches for several reasons, particularly for their safety, simplicity and convenience in introducing heterologous DNA into cells. Polyomavirus virus-like particles (VLPs) represent a promising carrier for encapsidation of foreign nucleic acids for gene therapy. For the development of such gene delivery systems as well as for providing reagents for improving virus diagnostics, an efficient yeast expression system for the generation of different polyomavirus VLPs was established. METHODS: A galactose-inducible Saccharomyces cerevisiae yeast expression system was used. Formation of empty VLPs was confirmed by cesium chloride ultracentrifugation, agarose gel electrophoresis and electron microscopy. Cross-reactivity of the major capsid proteins (VP1) of different polyomaviruses was analyzed by Western blot using rabbit and mice sera raised against the VP1 proteins. RESULTS: VP1 of polyomaviruses from humans (JC polyomavirus and serotypes AS and SB of BK polyomavirus), rhesus monkeys (simian virus 40), hamsters (hamster polyomavirus), mice (murine polyomavirus) and birds (budgerigar fledgling disease virus) were expressed at high levels in yeast. Empty VLPs formed by all yeast-expressed VP1 proteins were dissociated into pentamers and reassociated into VLPs by defined ion and pH conditions. Different patterns of cross-reactivity of the VP1 proteins with heterologous mice and rabbit sera were observed. CONCLUSION: The developed heterologous yeast expression system is suitable for high-level production of polyomavirus VLPs. Yeast-derived VLPs are generally free of toxins, host cell DNA and proteins. These VLPs might be useful for the generation of new diagnostical tools, gene delivery systems and antiviral vaccines.

PMID: 12602348 [PubMed - indexed for MEDLINE]

Anticancer Res. 2005 Jul-Aug;25(4):2601-8.

Murine polyomavirus virus-like particles (VLPs) as vectors for gene and immune therapy and vaccines against viral infections and cancer.

Tegerstedt K, Franzén AV, Andreasson K, Joneberg J, Heidari S, Ramqvist T, Dalianis T.

Department of Oncology-Pathology, Karolinska Institutet, Cancer Center Karolinska, R8: 01, Karolinska University Hospital, 171 76 Stockholm, Sweden.

This review describes the use of murine polyomavirus "virus-like" particles (MPyV-VLPs), free from viral genes, as vectors for gene and immune therapy and as vaccines. For large-scale MPyV-VLP manufacture, VP1 is produced in a baculovirus insect cell system, E. coli or in yeast. MPyV-VLPs bind eukaryotic DNA and introduce this DNA into various cell types in vitro and in vivo. In normal and T-cell-deficient mice, this results in the production of anti-MPyV-VLP (and MPyV) antibodies. Furthermore, repeated MPyV-VLP vaccination has been shown to prevent primary MPyV infection in normal and T-cell-deficient mice, and the outgrowth of some MPyV-induced tumours in normal mice. Moreover, when inoculated with gene constructs encoding for HIV p24, MPyV-VLPs augment the antibody response to p24. In addition, MPyV-VLPs, containing fusion proteins between the VP2 or VP3 capsid protein and selected antigens, can be used as vaccines. Notably, one vaccination with MPyV-VLPs, containing a fusion protein between VP2 and the extracellular and transmembrane parts of the HER-2/neu oncogene, immunizes against outgrowth of a HER-2/neu-expressing tumour in Balb/c mice and also against the development of mammary carcinomas in BALB-neuT transgenic mice. Finally, a second polyoma VLP-vector based on murine pneumotropic virus (MPtV-VLP), which does not cross-react serologically with MPyV-VLP (and MPyV), has been developed and can be used to conduct prime boost gene and immune therapy and vaccination. In summary, MPyV-VLPs are useful vectors for gene therapy, immune therapy and as vaccines and, in combination with MPyV-VLPs, MPtV-VLPs are potentially useful as prime-boost vectors.

PMID: 16080500 [PubMed - indexed for MEDLINE]

Curr Pharm Biotechnol. 2005 Feb;6(1):49-55.

Recombinant virus like particles as drug delivery system.

Georgens C, Weyermann J, Zimmer A.

Institute of Pharmaceutical Sciences, Karl-Franzens-University Graz, Schubertstrasse 6, 8010 Graz, Austria.

The drug delivery system described here is based on a virus like particle consisting of the recombinant expressed major capsid protein of Polyomavirus, VP1. Polyoma, a murine virus belonging to the Papovaviridae, forms a non-enveloped icosahedral capsid. These capsids are organized as a double shell composed of three different proteins: VP1,VP2 and VP3. The outer shell of the vision is composed of 360 VP1 molecules arranged as 72 pentamers. These capsids have a diameter of about 50 nm. The VP1 protein acts as a major ligand for certain membrane receptors during virus infection. Furthermore, the N-terminus of the VP1 protein contains a DNA-binding domain and a nuclear localization sequence. The recombinant production of the VP1 protein offers a save way to obtain a highly purified, non pathogenic pharmaceutical excipient. Combining these aspects, VP1 proteins provide a targeting as well as a drug binding site when used as a save drug carrier for gene therapy. Current applications are also including oligonucleotides as well as small molecules as well as vaccines.

PMID: 15727555 [PubMed - indexed for MEDLINE]

Gene Ther. 2000 Dec;7(24):2122-31.

Virus-like gene transfer into cells mediated by polyoma virus pseudocapsids.

Krauzewicz N, Stokrová J, Jenkins C, Elliott M, Higgins CF, Griffin BE.

MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Ducane Road, London, W12 0NN, UK.

Mouse polyoma virus-like particles (or pseudocapsids) are composed solely of recombinant viral coat protein. They can interact with DNA and transport it to cells, resulting in gene expression both in tissue culture and in mice. We demonstrate that DNA transfer in vitro depends on partial packaging of DNA within the virus-like capsid. Cell surface sialic acid residues and an intact microtubule network, required for viral infectivity, are also necessary for pseudocapsid-mediated gene expression from heterologous DNA. Thus, gene delivery in this system requires pathways utilised by polyoma virions, rather than proceeding via the 'nonspecific' endosomal route typical of nonviral systems such as liposomes or calcium phosphate precipitates. Despite the fact that all cells appear to internalise pseudocapsid/DNA complexes, only a proportion show productive gene delivery. Bulk internalisation of complexes is dependent on actin fibres, but not cell surface sialic acid or microtubules, indicating that a second transport pathway exists for pseudocapsids which is nonproductive for gene transfer. The model suggested by these data demonstrates the virus-like properties of the pseudocapsid system, and provides a basis for further development to produce a highly effective gene delivery vehicle. Gene Therapy (2000) 7, 2122-2131.

PMID: 11223994 [PubMed - indexed for MEDLINE]

Polyoma and Papilloma Virus Vectors For Cancer Gene Therapy

Book Series	Advances in Experimental Medicine and Biology
ISSN	0065-2598
Volume	Volume 465
Book	Cancer Gene Therapy
Publisher	Springer US
DOI	10.1007/b113267
Copyright	2000
ISBN	978-0-306-46191-0 (Print) 978-0-306-46817-9 (Online)
Part	II
DOI	10.1007/0-306-46817-4_8
Pages	73-82
Subject Collection	Medicine
SpringerLink Date	Tuesday, April 11, 2006

PDF (477.5 KB) Free Preview

Advances in Experimental Medicine and Biology

Cancer Gene Therapy
Past Achievements and Future Challenges

10.1007/0-306-46817-4_8

Nagy A. Habib

Nina Krauzewicz[7] and Beverly E. Griffin[7]

(7) Department of Infectious Diseases (7th Floor), Imperial College School of Medicine, Du Cane Road, London, W12 ONN, UK

Conclusions

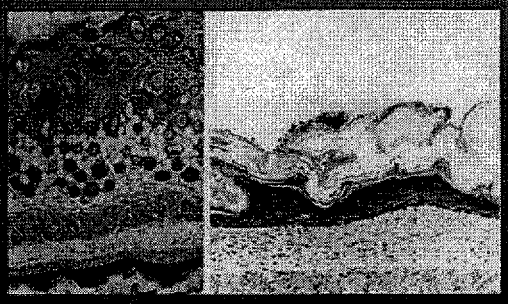
The studies carried out to date using polyoma and papillomavirus based carriers demonstrate their considerable promise, although numerous aspects of the systems require further attention. Present vectors vary widely in efficacy of DNA transfer, and parameters important in regulating this process need to be more clearly defined. Following establishment of a generalised prototype, modifications could then be made to target specifically to tumours, for instance by encoding tumour specific epitopes within the capsids, or crosslinking them to their surfaces. Further, delivery of prodrug activating enzyme genes to kill tumour cells, once targetted, could be explored. If it proves possible reliably to generate efficient and targetted pseudocapsid/VLP carriers, further approaches to anticancer therapy could be considered. For instance, delivery of wild type anti-oncogenes, such as p53, could be attempted, or where viruses (for example, papillo-maviruses, Epstein Barr virus, or herpesvirus-8) have been implicated in tumour formation, an approach aimed at inhibiting expression of identified oncogenic functions could be explored. These methods, coupled with stimulation of T cell killing by eliciting an immune response against tumour antigens expressed on the VLP/pseudocapsid surface, could produce a very efficient therapy vector.

METHODS IN MOLECULAR MEDICINE™

Gene Therapy Protocols

Edited by

Paul D. Robbins



Humana Press

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

This publication is printed on acid-free paper. ♾
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover illustration: Fig. 3 from Chapter 19, "Methods for the Use of Genetically Modified Keratinocytes in Gene Therapy" by Sabine A. Eming and Jeffrey R. Morgan.

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 201-256-1699; Fax: 201-256-8341; E-mail: humana@interramp.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods in molecular medicine™.

Gene therapy protocols/edited by Paul D. Robbins.
p. cm.—(Methods in molecular medicine™)
Includes index.
ISBN 0-89603-307-4 (combbound) (alk. paper); ISBN 0-89603-484-4 (hardcover) (alk. paper)
1. Gene therapy—methodology. I. Robbins, Paul D. II. Series.
[DNLM: 1. Gene therapy—methods. 2. Gene transfer. QZ 50 G3213 1997]
RB155.8.G465 1997
616'.042—dc20
DNLM/DLC
for Library of Congress 96-42147
CIP

7

Methods for the Construction of Human Papillomavirus Vectors

Saleem A. Khan and Francis M. Sverdrup

1. Introduction

A number of vector systems have been developed for the delivery of therapeutic genes into cells *(1)*. Many viral vectors suffer from the disadvantage of random integration into the chromosome, making the expression of the cloned genes dependent on the chromosomal context of the inserted DNA. Papillomaviruses (PVs) are potentially important vector systems because of their extrachromosomal replication in target cells. The PVs are small DNA viruses that infect humans and a wide range of animals. Human papillomaviruses (HPVs) induce benign proliferative squamous epithelial and fibroepithelial lesions (warts and papillomas) in their natural hosts *(2)*. Some HPVs are also involved in the pathogenesis of anogenital cancer and, in particular, cancer of the cervix *(2)*. Papillomaviruses contain circular, double-stranded DNA of approx 8 kb, and usually replicate extrachromosomally at a copy number estimated to be between 10 and 100 *(3)*. The potential advantages of PV vectors include expression of cloned genes from an extrachromosomal state that may be more amenable to uniform expression and possible elimination of problems associated with integration of DNA into transcriptionally inactive regions of the cellular chromosomes. Since PV DNA is not encapsidated, it may be possible to insert larger DNA sequences into such vectors, provided the DNA can still replicate in a stable manner. Bovine papillomavirus type 1 (BPV-1) vectors have been used to produce stable cell lines expressing foreign proteins (for reviews, *see* refs. *4–7*). The BPV-1 vectors used in these studies contained both replication and transforming genes, and in most cases, extrachromosomal replication of these vectors was accompanied by transformation of the target cells *(8–12)*. Recent advances in our understanding of the replication and transforming

From: *Methods in Molecular Medicine, Gene Therapy Protocols*
Edited by: P. Robbins Humana Press Inc., Totowa, NJ

genes of PVs have resulted in renewed efforts to develop vectors that can be established as stable extrachromosomal plasmids and express foreign proteins without oncogenic transformation of the host cell. The current article deals mostly with the potential of HPVs as vectors for gene therapy.

1.1. Organization of the HPV Genome

Most PVs have a similar genomic organization and their genome has been divided into the early and late regions. The early region includes eight ORFs (E1 to E8) involved in viral DNA replication, episomal maintenance, and cellular transformation *(5)*. The late region comprises genes that encode the viral structural proteins, L1 and L2 *(5)*. The E6 and E7 proteins of the high risk HPVs such as types 16 and 18 are involved in cell proliferation and tumor development *(2,13)*. A number of recent studies with PVs have shown the requirement for the viral E1 and E2 proteins in DNA replication *(14–19)*. The long control region (LCR) of PVs contains the promoter and enhancer sequences, including the binding sites for many viral and cellular factors. The LCR also contains the origin of replication (ori) of PVs *(3,18,19)*.

1.2. Replication of Human Papillomaviruses

The keratinocytes of stratified squamous epithelia are the natural hosts for HPVs. In benign papillomas and condylomas, HPV DNA replicates as a multicopy plasmid. The copy number of HPV is influenced by the differentiation state of the cell. In the basal cells of squamous epithelium, HPV DNA replicates at a low but stable copy number, whereas in the upper differentiating cells, vegetative replication occurs resulting in a high copy number. In the final stages of terminal differentiation, the late genes encoding structural proteins L1 and L2 are expressed. HPVs such as type 1a that cause common warts produce large amounts of virion and DNA, whereas types 16 and 18, which are predominantly associated with anogenital cancers, produce extremely low levels of DNA and virions. It is important to note that vegetative replication of HPV DNA is not required for their potential use as vectors for gene therapy. The PVs have been extensively studied in recent years for their replication properties. These studies have shown that the viral E1 and E2 proteins are both necessary and sufficient to support the transient replication of plasmids containing the ori of PVs *(14–19)*. In the case of HPV-1a, the E1 protein alone is sufficient for replication of ori-plasmids *(20)*. The ori of PVs contains an E1 binding site and multiple E2 binding sites. The E2 binding sites appear to act cooperatively to increase the efficiency of replication. We have also found that a region upstream of the HPV-1a origin appears to negatively regulate replication in transient assays *(20)*. The E1 protein of PVs is a nuclear phosphoprotein of approx 600 amino acids that has origin-binding, DNA helicase and ori-spe-

BMC Mol Biol. 2002 Apr 19;3:5. Epub 2002 Apr 19.

A bovine papillomavirus-1 based vector restores the function of the low-density lipoprotein receptor in the receptor-deficient CHO-ldlA7 cell line.

Tammur J, Sibul H, Ustav E, Ustav M, Metspalu A.

Institute of Molecular and Cell Biology, Tartu University, Estonian Biocentre, 23 Riia St, 51010 Tartu, Estonia.
jtammur@ebc.ee

BACKGROUND: The rationale of using bovine papillomavirus-1 (BPV-1) derived vectors in gene therapy protocols lies in their episomal maintenance at intermediate to high copy number, and stable, high-level expression of the gene products. We constructed the BPV-1 based vector harbouring the human low-density lipoprotein receptor (LDLR) gene cDNA and tested its ability to restore the function of the LDLR in the receptor-deficient cell line CHO-ldlA7. RESULTS: The introduced vector p3.7LDL produced functionally active LDL receptors in the receptor-deficient cell line CHO-ldlA7 during the 32-week period of observation as determined by the internalisation assay with the labelled LDL particles. CONCLUSION: Bovine papillomavirus type-1 (BPV-1)-derived vectors could be suitable for gene therapy due to their episomal maintenance at intermediate to high copy number and stable, high-level expression of the gene products. The constructed BPV-1 based vector p3.7LDL produced functionally active LDL receptors in the LDLR-deficient cell line CHO-ldlA7 during the 32-week period of observation. In vivo experiments should reveal, whether 1-5% transfection efficiency obtained in the current work is sufficient to bring about detectable and clinically significant lowering of the amount of circulating LDL cholesterol particles.

PMID: 11967145 [PubMed]

PMCID: PMC111063

Gene Ther Mol Biol Vol 9, 371-376, 2005

A Human Papillomavirus (HPV) - based pseudoviral gene delivery system for the non-viral, Episomally Replicating Vector pEPI-1

Research Article

Tobias Eisenberger[1,2], Hans Joachim Lipps[1], Martin Mueller[2,*], Armin Baiker[1,3,*]

[1]Institute of Cell Biology, University of Witten/Herdecke, 58452 Witten, Germany

[2]Programme Infection & Cancer, DKFZ Heidelberg, 69120 Heidelberg, Germany

[3]Max-von-Pettenkofer Institute, 80336 Munich, Germany

***Correspondence**: Armin Baiker, Ludwig-Maximilian University of Munich, Max-von-Pettenkofer Institute, Pettenkofer Strasse 9a, 80336 Munich, Germany; Phone: +49-89-5160-5289; Fax: +49-89-5160-5289; E-mail: <mailto:baiker@m3401.mpk.med.uni-muenchen.de>baiker@m3401.mpk.med.uni-muenchen.de and Martin Mueller, DKFZ Heidelberg, Programme Infection & Cancer, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany, Phone: +49-6221-424628, Fax: +49-6221-424932, E-mail: <mailto:Martin.Mueller@dkfz-heidelberg.de>Martin.Mueller@dkfz-heidelberg.de

Key words: pseudovirus; non-viral gene delivery; pEPI-1; episome; HPV 16

Abbreviations: Bovine Papillomavirus, (BPV); Episomally Replicating Vector, (pEPI-1); Epstein-Barr Virus, (EBV); Human Papillomavirus, (HPV); pEPI-pseudoviruses, (pEPI-PV); Pseudoviral particles harbouring pEPI-1, (pEPI-PV); scaffold/matrix attachment region, (S/MAR); Simian Virus 40, (SV40)

Received: 25 October 2005; Accepted: 10 November 2005; electronically published: November 2005

Summary

Non-viral episomal vector systems might allow a safe and reproducible genetic modification of eukaryotic cells and organisms. However, they share with all non-viral systems the problem of low efficient delivery to the target cells. To overcome this limitation we have utilized a Human Papillomavirus type 16 (HPV16)-based pseudoviral gene delivery system for the non-viral episomally replicating plasmid vector pEPI-1. Pseudoviral particles harbouring pEPI-1 (pEPI-PV) were prepared by co-transfection of the pEPI-1 plasmid DNA and a plasmid expressing the capsid proteins L1 and L2 of HPV16. Here we demonstrate that the resulting pEPI-PV were able to transduce target CHO cells with high efficiency under maintenance of the episomal character of pEPI-1. The combination of HPV-based pseudoviral gene delivery and non-viral, episomally replicating plasmids will provide a powerful new tool for biotechnical and possibly *in vivo* biomedical applications.

I. Introduction

In principle, non-viral, episomally replicating vectors could be considered most useful for the safe and reproducible genetic modification of mammalian cells and organisms and for *in vivo* gene therapy. These constructs are free from the problems related with currently used viral vector systems which include insertional mutagenesis, stimulation of the host immune system, or only transient expression of the transgene (Lipps et al, 2003; Glover et al, 2005). The prototype of such an episomal vector was recently constructed in our lab. This vector, pEPI-1 (**Figure 1A**), is 6700 bp in size, replicates at a copy number of around 5-10 molecules per stably transfected cell, does not integrate into the host cell genome, but remains episomally stable even in the absence of selection (Piechaczek et al, 1999; Baiker et al, 2000; Jenke et al, 2002). Recent work demonstrated that an upstream transcription unit directed into the scaffold/matrix attachment region (S/MAR) is necessary for its function (Stehle et al, 2003). These unique characteristics result in a very homogeneous protein expression pattern in transfected cells (Jenke et al, 2004a) making it a valuable tool for the reproducible analysis of candidate genes eliminating the need for screening a vast number of clones. Due to its extrachromosomal replication mechanism pEPI-1 derived vectors do not cause insertional mutagenesis as known from retroviral and lentiviral vector systems (Marshall, 2000; Check, 2002). Furthermore pEPI-1-based vectors do not require any viral trans-activating proteins for their function, such as the

Mol Cell Biol. 1981 Jun;1(6):486-96.

Bovine papilloma virus deoxyribonucleic acid: a novel eucaryotic cloning vector.

Sarver N, Gruss P, Law MF, Khoury G, Howley PM.

Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20205.

A novel eucaryotic vector derived from the transforming region of bovine papilloma virus was established and demonstrated to be highly effective for introducing foreign genes into animal cells. The foreign deoxyribonucleic acid (DNA) is replicated and actively transcribed as an episome, and the transcripts are translated into an authentic gene product. We have constructed a DNA hybrid molecule, BPV69T-rI1, containing the transforming region of bovine papilloma virus DNA and the rat preproinsulin gene I (rI1), and used it to transform susceptible mouse cells. DNA hybridization analysis has demonstrated the presence of multiple unintegrated copies of hybrid DNA molecules, with the bovine papilloma virus 1 DNA segment and the rI1 gene covalently linked in selected transformed cell lines. S1 nuclease analysis revealed the presence of a correctly spliced coding segment of the preproinsulin transcript similar or identical in its electrophoretic mobility to that of messenger ribonucleic acid produced in rat insulinoma cells. Significant levels of a protein immunoreactive with anti-insulin serum were detected by radioimmunoassay in the culture medium of transformed cells. Immunoprecipitation analysis in conjunction with competitive binding to bovine proinsulin established the identity of the protein as that of rat proinsulin.

PMID: 6100967 [PubMed - indexed for MEDLINE]

PMCID: PMC369692